JOURNAL OF VIROLOGY, Aug. 2005, p. 9896–9903 Vol. 79, No. 15
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.15.9896–9903.2005

Age-Related Differences in Amplification of Covalently Closed Circular DNA at Early Times after Duck Hepatitis B Virus Infection of Ducks

Yong-Yuan Zhang,* Daniel P. Theele, and Jesse Summers

Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

Received 10 February 2005/Accepted 1 April 2005

Inoculation of 3-day-old (3D) or 3-week-old (3W) ducklings with duck hepatitis B virus results in chronic or transient infection, respectively. We previously showed that rapid production of neutralizing antibody following inoculation of 3W ducklings prevents virus from spreading in the liver and leads to a transient infection (Y.-Y. Zhang and J. Summers, J. Virol. 78:1195–1201, 2004). In this study we further investigated early events of viral infection in both 3D and 3W ducks. We present evidence that a lower level of virus replication in the hepatocytes of 3W birds is an additional factor that probably favors transient infection. We suggest that lower virus replication is due to a less rapid covalently closed circular DNA amplification, leading to lower viremias and a slower spread of infection in the liver, and that the slower spread of infection in 3W ducks makes the infection more sensitive to interruption by the host immune responses.

Hepadnaviruses are small DNA viruses that cause both transient and persistent infections of the liver (2, 21). The prototype virus of this family is the human hepatitis B virus (HBV), a cause of chronic liver disease, including hepatocellular carcinoma (3). Duck hepatitis B virus (DHBV), a member of this family, is a model virus for studying HBV replication and infection (11, 13, 22). Initiation of hepadnavirus infection begins with the formation of covalently closed circular DNA (cccDNA) in the nucleus (12, 15, 19, 25). cccDNA serves as the template molecule for viral transcription (21). Viral RNA transcribed from cccDNA is transferred to the cytoplasm where viral proteins are synthesized and new viral relaxed circular DNA (rcDNA) is subsequently produced by reverse transcription (21, 26). The newly synthesized rcDNA can be directed to the nucleus to amplify or replenish the cccDNA pool (25, 27), or it can be directed into a virus assembly pathway (7). The usage of these alternative pathways for newly synthesized rcDNA is regulated by the production of viral pre-S proteins (7, 23, 24). The size of the cccDNA pool has an important impact on the level of viral replication, since viral gene expression is controlled at one level by the gene dosage, the number of cccDNA copies in each nucleus (24).

Transient or persistent infection with HBV is largely determined by the age at which the infection is acquired. When the viral infection occurs perinatally, more than 90% of such infections become persistent (1, 16, 20). In contrast, more than 90% of infections occurring in adults are transient (14, 18). A similar relationship between the age at which infection occurs and persistence of the infection is seen in ducks infected with DHBV (4–6). The mechanisms responsible for age-related outcome of hepadnavirus infection are not clear. It appears that the spread of the infection from a small fraction of initially infected cells to the whole liver or the restriction of the infection to the initially infected cells is a key step for the determination of different outcomes. We have shown that rapid production of neutralizing antibody in 3-week-old (3W) ducks at early times of the infection blocks the spread of virus in the liver and leads to a transient infection (30). The effectiveness of blocking the spread of the infection by neutralizing antibody probably depends on the amount of available neutralizing antibody and the level of viremia. Therefore, the level of virus production at the earliest stage of infection may determine the effectiveness of the neutralizing antibody and influence whether an infection spreads throughout the liver.

In this study we investigated the level of replication and production of virus immediately after infection in 3-day-old (3D) and 3W DHBV-infected ducks. We found that the viremia at 2 days postinoculation (p.i.) in the 3D group was 10- to 500-fold higher than that in the 3W group, even though the fractions of initially infected cells were similar in both groups. Measurements of replicative intermediates and cccDNA at 1 day p.i. suggested that rcDNA was converted to cccDNA more rapidly in 3D ducks than in 3W ducks, resulting in higher rates of virus production and spread of the infection.

MATERIALS AND METHODS

Animals, virus inocula, and sample collection (Table 1). One-day-old Peking ducklings were purchased from Metzer Farms (Redlands, Calif.). Congenitally DHBV-infected ducklings, detected after screening by dot blotting (9), were excluded from experiments. A pooled serum-derived DHBV that was collected from a congenitally infected Peking duck during a period of 2 weeks (3 days to 20 days old) was used as the inoculum. Viral DNA was extracted from 10 μl pooled serum in duplicate (see below), and the virus titer of the pooled serum was determined by real-time PCR. The procedure used for DNA extraction from the serum was published previously (28). Two groups of ducks at different ages were used for DHBV infection. One group was infected at 3 days after hatching (3D group), and the other group was infected at 3 weeks after hatching (3W group). Each 3-day-old and 3-week-old duck was intravenously inoculated with 1.6×10^8 and 1.6×10^9 viral genome equivalents, respectively, in 0.2- to 0.3-ml

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, MSC 08 4660, Albuquerque, NM 87131. Phone: (505) 272-8895. Fax: (505) 272-9494. E-mail: yzhang@salud.unm.edu.

TABLE 1. Ducks used in the experiments

Age group	Animal no.	Maternal antibody present[a]	Inoculum dose	Time of biopsy (day)	Time of early bleeds (h)	Time sacrificed
3D	51, 52, 55, 57, 59, 60, 63, 64	52, 59, 63	1.6×10^8	1	0, 1, 3, 6	8 days
	53, 54, 56, 58, 62	54	1.6×10^8			8 days
3W	13a, 14a, 17a, 18a, 19a	ND	1.6×10^9			1.5–3.5 h
	15, 16, 18, 19, 20, 22, 63a, 67, 72, 73	ND	1.6×10^9	1	0, 1, 3, 5	8 days

[a] Blocking of infection of primary duck hepatocytes by serum samples from these birds. nd, not detected.

volume. The experimental infection of the 3W group was conducted three times, each time with four to six ducks. Serum samples were collected from each animal for determining the presence or absence of the maternal antibody before the inoculation.

Blood samples at 5 min, 1, 3, and 5 or 6 h p.i. were drawn from each animal for determining kinetics of inoculated viruses in the plasma. Then serum samples were collected daily from day 1 to day 8 p.i. for monitoring viremia in each animal. Liver biopsy was performed in five 3W ducks at 1.5 to 3.5 h p.i. for determining the amount of inoculum virus bound in the liver. Liver biopsies were then performed in the rest of ducks at day 1 p.i.

Extraction of DNA from serum for determining viremia and titers of inoculated viruses. Ten microliters of serum was mixed with 352 μl TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5) containing 0.2% sodium dodecyl sulfate, 500 μg/ml pronase (Calbiochem), 20 μg tRNA (Roche), and 150 mg NaCl/ml and incubated at 37°C for 1 hour. Then digested serum was subjected to phenol extraction and ethanol precipitation. DNA was dissolved in 20 μl TE buffer with 1 μg/ml RNase A (Sigma) and incubated at 37°C for 10 min. An amount of DNA equivalent to the amount in 0.5 μl serum was used for real-time PCR.

Isolation of replicative intermediates and cccDNA from liver biopsies. About 30 mg liver tissue was homogenized in the presence of 1 ml cold buffer consisting of 150 mM Tris-HCl and 1 mM EDTA. Cytoplasmic and nuclear fractions were separated from the homogenate by microcentrifugation. Extraction of replicative intermediates from the supernatant follows the protocol developed by this laboratory (8). The pellet portion was used for isolation of cccDNA after alkaline denaturation, potassium acetate precipitation, and phenol extraction at pH 5 (10).

Determining copy numbers of cccDNA and replicative intermediates by real-time PCR. Purified cccDNA was subjected to EcoRI digestion before PCR amplification. The amplification of cccDNA molecules was conducted with a pair of primers that flank the cohesive region (DHBV 2407 [5′ TGT CCC GAG CAA ATA TAA TCC] and DHBV 2840R [5′ TGT GTA GTC TGC CAG AAG TCT TC]). The specificity for amplification of cccDNA was tested by comparing EcoRI-cut templates with uncut templates. The detection of cccDNA was increased by 40- to 120-fold by EcoRI cleavage compared with uncut template (data not shown). Because only linearized cccDNA can be denatured by heat to allow PCR amplification, this result suggested that the overwhelming majority of templates amplified by this primer set after digestion were derived from cccDNA.

For real-time PCR analysis of copy number of cccDNA and replicative intermediates, 5 μl extracted replicative intermediates or EcoRI-cleaved cccDNA was mixed with 10 μl 2× SYBR green supermix (Bio-Rad), primers, and water in a total volume of 20 μl. The PCR mix contained a final concentration of 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 200 μM of each deoxynucleoside triphosphate, 0.5 unit of iTaq DNA polymerase, 3 mM $MgCl_2$, SYBR Green I, 10 nM fluorescein, and 200 nM of each primer (DHBV2548 [5′ TTC GGA GCT GCT TGC CAA GGT ATC] and DHBV2840R were used for detection of replicative intermediates). The PCR amplification cycle consisted of three temperature profiles: denaturation at 94°C for 20 s, annealing at 58°C for 15 s, and elongation at 72°C for 45 s. The specificity of PCR products was verified by melting curves or agarose electrophoresis. In order to have accurate standard plasmid controls, the supercoiled plasmid containing the DHBV genome was isolated from an agarose gel and recovered using a commercial gel purification column (QIAGEN). The optical density at 260 nm was measured to determine the copy number, and the purified plasmid was linearized by EcoRI cleavage.

Isolation and distribution of nuclei. Nuclei were isolated as previously described (29). The purified nuclei were suspended with buffer containing 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 0.25 M sucrose, 0.05% Triton X-100, and ethidium bromide (1 μg/ml), counted with a hemacytometer, and diluted to a concentration of 10 nuclei/5 μl. Then 5 μl of this suspension was distributed into each one of the 96 wells of the PCR plate; each well contained 5 μl/well proteinase K solution (500 μg/ml). Nuclei in the wells of the PCR plate were incubated for 1 hour at 50°C to release cccDNA and then for 15 min at 75°C to inactivate proteinase K. Then 10 μl PCR mix (containing 2 μl of the supplier's PCR buffer [Roche], 200 μM each deoxynucleotide triphosphate, 2 units of EcoRI, 0.7 unit of Expand High Fidelity DNA polymerase [Roche], 375 nM each primer [DHBV2407 and 2840R] that flank cohesive ends of the DHBV genome) was added to each well. The nuclei were subjected to EcoRI digestion and the first PCR, which consisted of 37°C for 60 min, denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 45 s. The second PCR was performed as described below.

Measurement of cccDNA copy in each infected cell. Briefly, all the DNA released by proteinase K digestion was cut with EcoRI and diluted to 120 μl with a complete PCR mixture containing (final amounts) 12 pmol each of primers (DHBV2407 and 2840R), 4.2 units Expand High Fidelity DNA polymerase (Roche Diagnostics Corp.), and 12 μl of the supplier's PCR buffer containing 15 mM $MgCl_2$. The PCR mix was distributed in 10-μl aliquots to 12 wells of a PCR microplate and subjected to the following amplification: denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 20 seconds, 58°C for 15 seconds, and 72°C for 45 seconds, and a final elongation step of 72°C for 4 min. For the second PCR, approximately 0.1 μl of each reaction mixture was transferred to a new PCR plate with reaction mixtures that contained a heminested primer set. Seeding of the nested reactions was carried out by use of a 96-well microplate pin replicator (Nalgene Nunc International Corp., Naperville, IL). The heminested reaction mixture in a volume of 10 μl consisted of 5 pmol each of primers specific for cccDNA (DHBV2437 [5′ CCC ATC CAG GCA CAA A] and DHBV2840R), 200 μM each deoxynucleoside triphosphate, 0.25 unit Ampli Taq Gold DNA polymerase (Applied Biosystem), and 1 μl of the supplier's 10× PCR mix containing 2.5 mM $MgCl_2$/ml.

Assay for maternal antibody by blocking infectivity. The assay for maternal antibody was done using primary duck hepatocyte (PDH) cultures. Preparation of hepatocyte cultures followed the protocol previously described (17). To determine whether a particular serum sample contained maternal antibody, an inoculum of 2×10^6 virus particles was first mixed with 100 μl serum and incubated at 37°C for 1 hour and then added to PDH cultures 1 day after plating. The medium was changed daily. Cultures were harvested for DNA extraction at 9 days postinoculation. Blocking of infectivity was judged by comparing the amount of replicative intermediates in PDH cultures infected with the standard inoculum alone or the inoculum incubated with fetal bovine serum or with a test serum sample.

RESULTS

We have observed that titers of DHBV in experimentally infected 3D ducklings are significantly higher than those in 3W ducks in the first days postinfection. The initial rate of virus production could affect how fast the infection could be spread in the liver. We investigated factors that could lead to high rates of virus production in the 3D ducklings with DHBV infection: kinetics of uptake of inoculated virus, the number of initially infected cells, and the extent of amplification of the cccDNA pool.

Kinetics of inoculated viruses and the influence of maternal antibody. The presence of maternal antibody in serum could have an effect on the number of hepatocytes that are initially infected. Maternal antibody in the preinfected serum samples

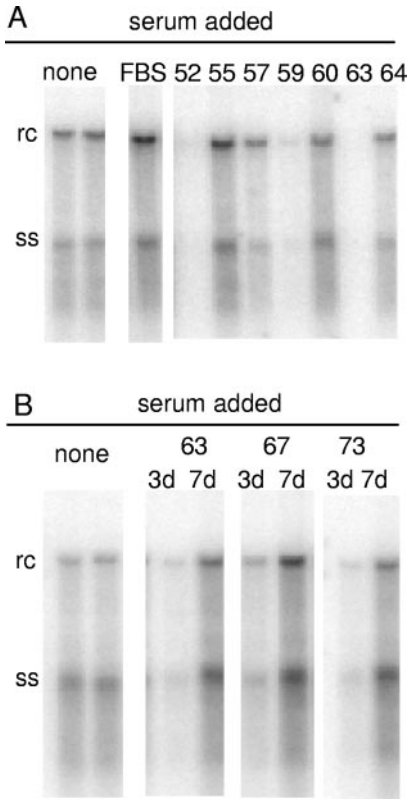

FIG. 1. Detection of maternal antibody present in preinfected serum samples. Preinfection serum samples from 3D and 3W ducks were analyzed for their ability to block infection of primary duck hepatocytes with 2×10^6 virus particles. After incubation with 100-μl preinfection serum samples from the indicated ducklings (ducks 52, 55, 57, 59, 60, 63, 64, and 73), the inoculum was added to cultures of primary duck hepatocytes. At 9 days postinfection, viral replicative intermediates were extracted and analyzed by Southern blotting. A reduction of replicative intermediates compared with inoculum alone (none) or inoculum incubated with fetal bovine serum (FBS) was taken to indicate the presence of the maternal antibody. (A) 3D group; (B) 3W group. Note that only serum samples collected from three ducks at ages 3 and 7 days (3d and 7d, respectively) of the 3W group are shown, since blocking activity was found to be absent by 7 days after hatching. The positions of rcDNA (rc) and single-stranded DNA (ss) are shown to the left of the blots.

from both groups was assayed by its ability to block infection of duck primary hepatocyte cultures by a standard inoculum. Blocking antibody could be detected in some serum samples from 3-day-old ducklings, but this activity disappeared at later times and was not detected after 7 days (Fig. 1 and Table 1).

The kinetics of disappearance of inoculated virus from serum were fairly uniform among the different ducks in the 3W group (Fig. 2). The titers of inoculated viruses were around 2×10^7 viral genomes/ml in sera taken at 5 min p.i. and only 10^4 to 10^3 viral genomes/ml at 5 h after inoculation. In contrast, the kinetics of disappearance of the inoculated viruses in the sera of the 3D group varied from duck to duck. For example, the titers of the inoculated viruses in the sera taken at 5 min p.i. differed over a 100-fold range, and the rate of loss of virus from serum was variable and more rapid in several of the ducks from the 3D group (Fig. 2). Rapid disappearance of the inoculated virus was correlated with the presence of maternal antibody in the 3D group (Table 1); i.e., 3D ducklings showing the lowest initial titers of inoculated viruses (ducks 52, 54, 59, and 63) and complete removal within 1 hour had the highest activities of maternal antibody (Fig. 1). The accelerated removal of inoculated virus from blood was associated with a reduced number of infected cells detected at day 1 p.i. (see below).

Uptake of inoculated virus by the liver. To determine the amount of virus that localized to the liver during the disappearance of virus from blood, five birds from the 3W group were sacrificed between 1.5 and 3.5 h p.i., and total viral DNA was extracted from pieces of liver and quantified by real-time PCR. The amount of viral DNA was normalized to the number of liver cells assayed, as determined by counting nuclei in the homogenized tissue before extraction. As shown in Table 2, the amount of viral DNA ranged from 0.02 to 0.09 molecule per cell. This amount of viral DNA accounted for 3 to 11% of the total number of viral genomes injected in the inoculum. Since the liver constitutes about 5% of the body mass of young ducklings, our results fail to demonstrate liver-specific homing of inoculated virus. The livers from birds in the 3D group were not analyzed in this manner.

Fraction of infected cells at 1 day p.i. We analyzed the fraction of liver cells initially infected with inoculated virus in 8 ducklings of the 3D group and in 10 ducklings of the 3W group. Infection was determined by the presence of cccDNA, detected by PCR in dilutions of nuclei isolated from liver biopsies taken at 1 day p.i. At dilutions of nuclei that yielded a small fraction of reactions positive for cccDNA, we assumed that each cccDNA-positive well was derived from one infected nucleus. By this assay, we estimated that 1.3 to 4.2% of nuclei were infected in the 3W group ducks. In the 3D group, the ducklings with maternal antibody showed very low to undetectable levels of infection (ducks 52, 59, and 63), while in ducklings with no maternal antibody, 1.3 to 4.7% of nuclei were infected (Table 3). These data suggest that maternal antibody could greatly influence the efficiency at which a viral inoculum established infection, but otherwise, infection of hepatocytes was initiated with similar efficiencies in the 3D and 3W groups.

Replicative intermediates and cccDNA molecules in the livers at 1 day p.i. Total replicative intermediates and cccDNA from the same piece of liver tissue taken at 1 day p.i. were assayed by real-time PCR. The copy numbers of replicative intermediates and cccDNA were normalized to the number of infected cells analyzed, estimated from the number of cells extracted and the fraction of cells infected. As shown in Table 3, the average copy numbers of both replicative intermediates and cccDNA per infected cell were higher in the 3D group (15.1 and 6.0, respectively) than in the 3W group (7.7 and 1.3, respectively). This result suggests that cccDNA may amplify more rapidly in 3D ducklings than in the 3W ducks.

To compare more directly the cccDNA copy numbers per cell between the two groups of ducks, individual nuclei were analyzed for the number of molecules of cccDNA. Dilutions of nuclei containing a single cccDNA molecule were extracted, and the cccDNA molecules were distributed among 6 or 12 wells of a PCR microplate, and each well was analyzed for the presence of a cccDNA template by nested PCR. As shown in Table 4, out of 15 infected nuclei assayed, 13 nuclei yielded only one positive well, suggesting the presence of a single cccDNA molecule, while the remaining two nuclei yielded two

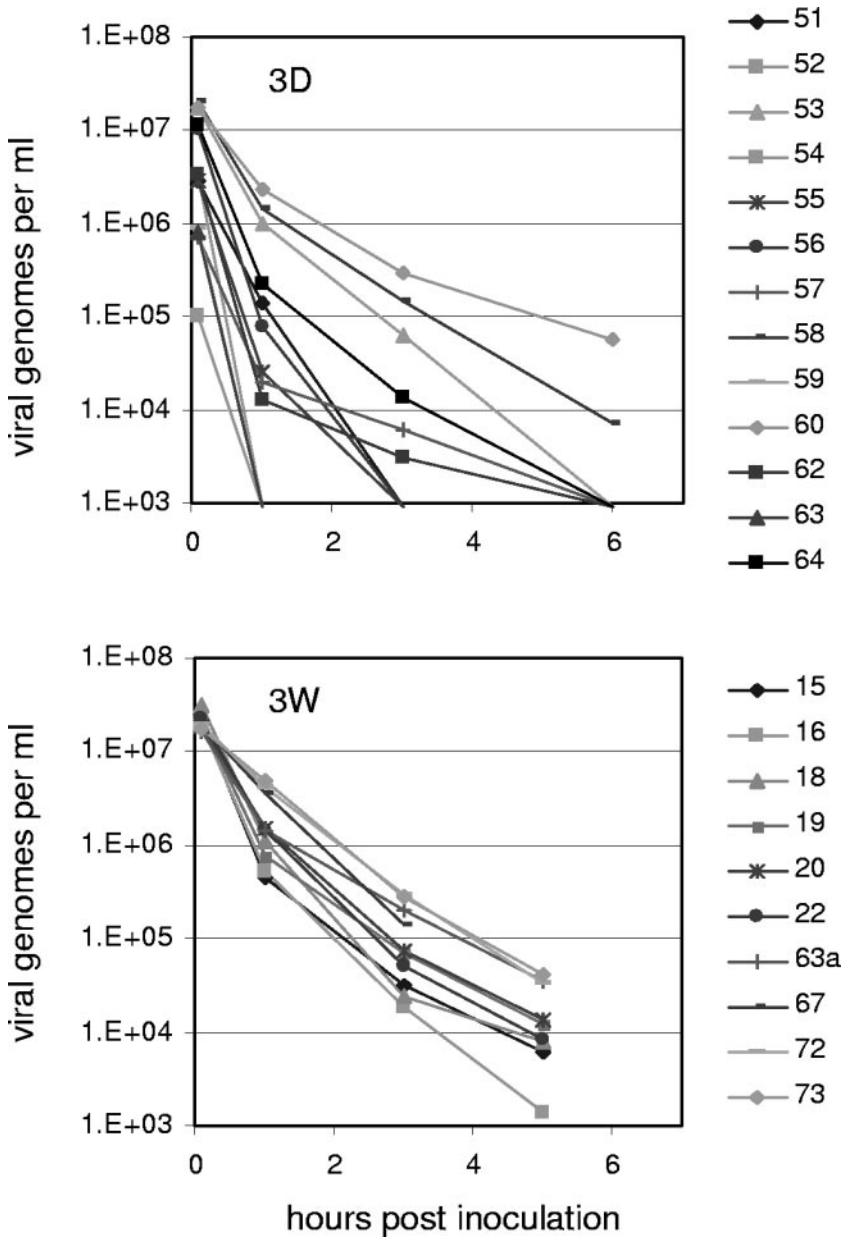

FIG. 2. Kinetics of disappearance of inoculated virus from the blood. Serial serum samples (10 μl) from each animal obtained at 5 min, 1, 3, and 5 or 6 h postinoculation were extracted, and DNA equivalent to the amount of DNA in 0.5 μl serum was amplified in duplicate by real-time PCR. Data from individual ducks are shown.

positive wells. In contrast, of 20 infected nuclei from the 3D group, 12 nuclei yielded 1 or 2 positive wells and 8 nuclei yielded 3 to 12 positive wells. The number of cccDNA molecules per nucleus ranged from 1 to 2.4, with a mean of 1.3, in the 3W group. The number ranged from 1 to 29 or more, with a mean of 6.4 or more in the 3D group. These numbers are in good agreement with the average estimates in Table 3, but in addition, they reveal that the higher average copy numbers in the 3D group were due to the large contributions of a subset of hepatocytes.

Viremia at day 2 p.i. Viremia was not detected at 1 day p.i. in either group. However, at 2 days p.i. viremia was detected in both groups, and the mean viremia was approximately 17-fold

TABLE 2. Recovery of inoculum in the livers of 3W ducks

Duck no.	Time p.i. (h)	No. of genomes/ liver cell	Body (liver) mass (g)[a]	Fraction of inoculum recovered in liver[b]
13a	1.5	0.05	870 (44)	0.07
14a	2.0	0.08	790 (40)	0.10
17a	2.5	0.09	790 (40)	0.11
18a	3.0	0.07	840 (42)	0.09
19a	3.5	0.02	880 (44)	0.03

[a] Liver mass calculated as 5% of body mass.
[b] Calculated as follows: (number of genomes per liver cell)(5×10^7 cells) (number of grams of liver)/(1.6×10^9).

TABLE 3. Assays for replicative intermediates, cccDNA, and viremia

Age group	Animal no.	% Infected cells	Copy no. of:			
			RI/infected cell at day 1[a]	cccDNA/infected cell at day 1	Virus at day 2 (10^{-5})	Viral DNA/hepatocyte at day 8
3D	51	4	9.6	5.1	80	1,304
	52	<0.1	ND[b]	ND		
	55	3.9	23.3	7.6	100	982
	57	1.3	6.3	3.5	9	1,349
	59	0.1	20.2	10.0	0.2	1,171
	60	4.7	15.7	5.1	90	980
	63	<0.1	ND	ND		
	64	1.3	15.5	4.9	20	759
	Mean	2.6 ± 1.9	15.1 ± 6.4	6.0 ± 2.4	50 ± 50	1,090 ± 225
3W	15	2.6	6.9	1.1	0.9	187
	16	2.8	7.5	1.1	5	2,107
	18	2.6	25.5	2.9	9	1,212
	19	3.3	11.9	2.1	5	827
	20	2.9	6.7	1.5	1	1,415
	22	4.2	5.7	0.4	4	1,813
	63a	1.2	2.2	1.0	0.3	704
	67	1.6	1.6	1.1	0.2	234
	72	1.3	6.2	0.8	0.6	10
	73	3.2	2.9	1.0	2	1,655
	Mean	2.6 ± 1.0	7.7 ± 6.9	1.3 ± 0.7	3 ± 3	1,016 ± 736

[a] RI, replicative intermediates.
[b] ND, not detected.

higher in 3D ducks than in 3W ducks (Table 3). We observed a rough correlation between the fraction of infected cells and the viremia at day 2 in individuals of both age groups; however, the 3D ducks on average produced a higher viremia per infected cell than did the 3W ducks, consistent with their higher copy numbers of cccDNA.

Rate of spread of the infection among 3D and 3W ducks. As shown in Fig. 3, the increase in viremia in 3D ducks approximated first-order kinetics, reaching a maximum at around 10^{10} viral genomes per ml. The rate of increase in virus titers was independent of the titer at day 2 in all animals except one (duck 63) in which a high activity of maternal antibody strongly reduced the level of infection and apparently prevented its spread. These data indicated that absent strong maternal antibody sufficient to block infection and/or spread completely, the time for complete spread of infection depended primarily on the initial number of infected cells. The levels of viral replicative intermediates in the livers of the 3D group at sacrifice 8 days p.i. were similar for all animals (mean, 1,090 ± 225 molecules/cell; range, 759 to 1,349 molecules/cell).

In contrast, there was no correlation in the 3W group between the levels of viremia at 2 days p.i. and subsequent levels of viremia. The amounts of virus in blood in this age group rose or fell sporadically, regardless of the initial level of infection or viremia at day 2 (Fig. 3). In addition, we found that the amount of viral DNA in the liver at sacrifice at 8 days p.i. differed among individual ducks of the 3W age group as much as 200-fold (mean, 1,016 ± 736 molecules/cell; range, 10 to 2,107molecules/cell), and these amounts did not correlate with the individual viremias at the time of sacrifice. Immunohistochemical detection of the DHBV pre-S/S protein (not shown)

TABLE 4. Assay for cccDNA copy numbers in single nuclei at day 1 postinfection

Age group and duck	No. of:		
	Positive wells	Expected cccDNAs/nucleus[a]	Nuclei
3D duck 55	1	1.0	8
	2	2.2	4
	3	3.5	1
	4	4.9	0
	5	6.5	2
	6	8.3	1
	7	10.5	1
	8	13.2	0
	9	16.6	1
	10	21.5	0
	11	29.8	1
	12	>29.8	1
	Mean	>6.4	
	Total no.		20
3W ducks 72 and 73	1	1.1	13
	2	2.4	2
	3	4.2	0
	4	6.6	0
	5	10.8	0
	6	>10.8	0
	Mean	1.3	
	Total no.		15

[a] From Poisson distribution, $-\ln(P(0)) \times$ number of wells.

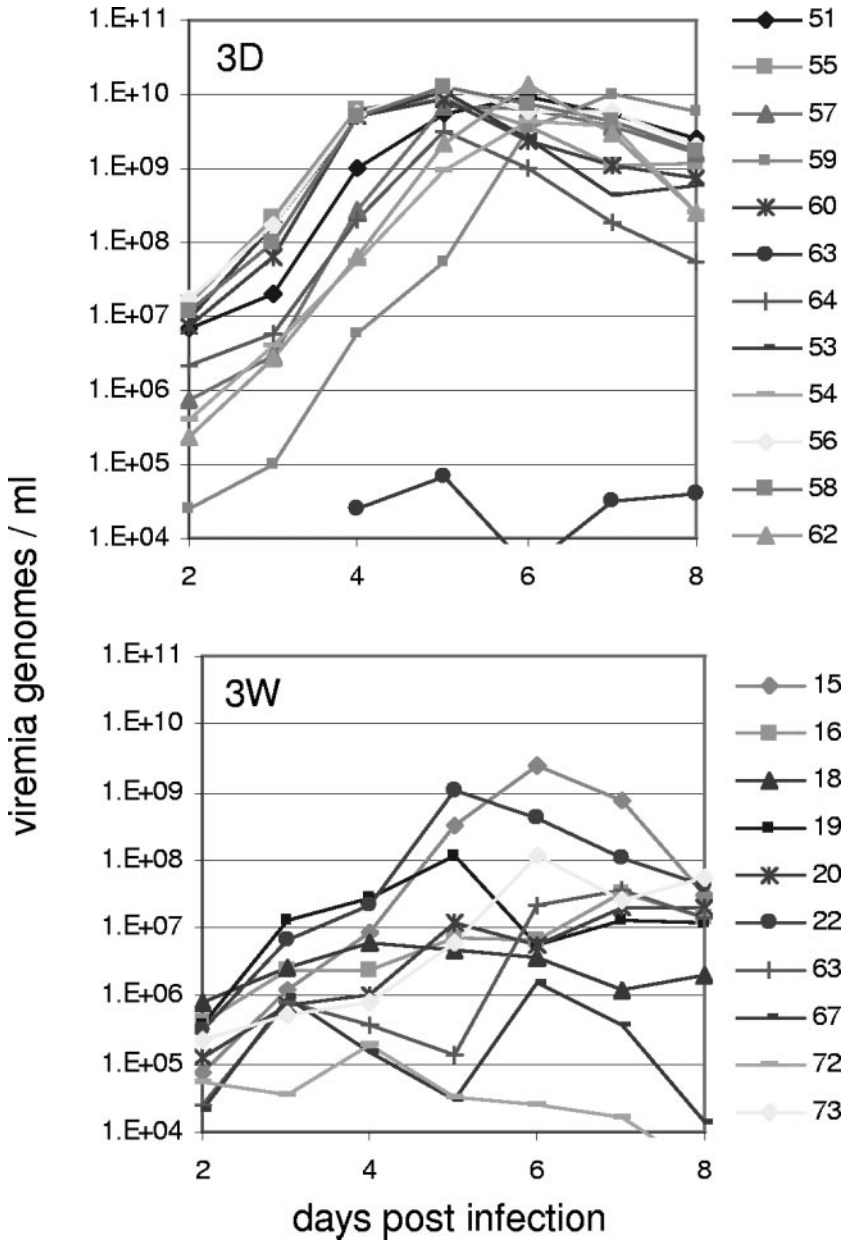

FIG. 3. Accumulation of virus in the blood of 3D and 3W ducklings. Serial serum samples were collected daily from each animal. DNA was extracted from 10 μl serum at each time point, and an amount equivalent to 0.5 μl was assayed in duplicate by real-time PCR. Data from individual ducks are shown.

indicated that the differences in viral DNA in the livers of the ducks were at least partially due to the number of infected hepatocytes, with values of 700 copies per cell or more corresponding to infection of all hepatocytes. We do not know whether the differences in the extent of infection were due to a failure of the infection to spread throughout the liver or whether some infections were in the process of clearance at the time of sacrifice. In a previous study in which 3-week-old ducklings were infected with a lower dose of virus (30), we showed that a rapid production of neutralizing antibody prevented the spread of infection throughout the liver. If the fluctuating levels of viremia in the 3W group were similarly due to antibody responses, they were insufficient in at least 7 of 10 birds, resulting in a complete block of the spread of infection.

DISCUSSION

3D ducklings differ from 3W ducklings in their susceptibility to DHBV infection. In the 3D birds, virus infection spreads rapidly from very low doses to produce a fully infected liver that usually persists through the lifetime of the duck. In 3W ducks, the outcome of an infection depends on the size of the inoculum, with smaller inocula generally resulting in a transient infection that does not spread through the liver (6). We

previously demonstrated that rapid production of neutralizing antibody in 3W ducks, either in response to the inoculum or to the initially infected cells, is associated with failure of low-dose infections to spread (30). In this study we examined whether other differences in early events in infection of 3D and 3W ducklings might also influence the outcome.

Kinetics of uptake of the inoculum. The disappearance of virus from blood in ducks from the 3W group was observed to occur at similar rates in all birds. However, at the first bleed, which was taken at approximately 5 min p.i., the amount of virus in blood accounted for only 10 percent or less of the inoculum injected. This suggests that 90% or more of the added inoculum was taken up from the blood within 5 min. Moreover, after viral clearance from the blood, between 1.5 to 3.5 h after inoculation, the amount of viral genomes recovered from the liver accounted for only about 2 to 11% of the inoculum. Therefore, most viruses were removed from the blood by pathways that did not result in binding to liver cells or their infection. The amount of virus taken up the liver, however, corresponded approximately to the number of infected cells determined by the presence of cccDNA in liver cell nuclei at day 1 p.i. in ducks infected with the same inoculum. This result indicated that viral infection was successfully established in almost every cell in the liver with bound virus.

In the 3D group of ducks, the rate of removal of virus from the blood over the first 6 h after inoculation differed significantly from bird to bird, and the amount of virus present at 5 min was already low in those individuals with higher rates of viral clearance. This enhanced rate of virus uptake from the blood was most evident in birds with demonstrable maternal antibody (ducks 52, 59, and 63), suggesting that accelerated removal was due to the presence of maternal antibody, perhaps by opsonization of antibody-bound virus. These birds also had significantly reduced numbers of infected cells at day 1 p.i., either because removal from the blood prevented infection or because antibody binding neutralized infectivity of the virus or both. Aside from the influence of maternal antibody in 3D ducklings, no consistent differences were observed in the efficiencies of infection of 3D and 3W ducklings.

Viral DNA replication at early times of infection. At 1 day p.i. infected hepatocytes in the 3D ducklings appeared to have progressed to a more advanced stage of infection than those in the 3W group, as evidenced by a higher number of copies of both replicative intermediates and cccDNA. However, hepatocytes from 3W birds contained more replicative intermediates per cccDNA than those from 3D birds. This distribution is consistent with a reduced rate of conversion of rcDNA to cccDNA resulting in a lower rate of cccDNA amplification and virus production. Assay of cccDNA levels in individual nuclei indicated a broad range of cccDNA amplification levels in 3D, but not in 3W, ducklings. Whether this range reflects differences in the intrinsic ability of hepatocytes to amplify cccDNA is not known.

The factors that influence the rate of cccDNA amplification are not entirely defined. Pre-S envelope protein is known to inhibit cccDNA synthesis (7, 23, 24), and it is possible that excess early production of this viral protein from the initial cccDNA in 3W hepatocytes could cause the low levels observed. Cell-type-specific factors are also certainly involved, as cccDNA synthesis does not occur in many cell types transfected in vitro (J. Summers, unpublished observation). It is possible that developmental or adaptive factors directly influence the ability of DHBV to establish an infection in hepatocytes by affecting the rate of cccDNA amplification.

In both 3D and 3W ducks, the viremia observed at 2 days p.i. appeared to be directly related to the number of infected cells and the amount of cccDNA per cell. Thus, major differences in the earliest stages in the spread of infection are primarily determined by the absence of maternal antibody and the age-dependent ability of cells to amplify cccDNA.

Factors determining the rate of spread of infection in 3D and 3W groups. The rate of spread of infection in 3D ducklings was much more uniform and generally higher than that seen in 3W ducks, even when the numbers of initially infected cells were affected by maternal antibody. As shown in Fig. 3, the accumulation of virus in blood followed approximately the same kinetics (doubling time about 7 h), regardless of the starting level of viremia at day 2 p.i. This result indicates that virus spread proceeded unimpeded from the initially infected cells in 3D ducklings. However, in 3W ducks the accumulation of virus in blood was variable from bird to bird and was not correlated with viremia at 2 days p.i. (Fig. 3). Lack of such correlation indicates that there are factors in addition to the initial number of infected cells that determine the course of the infection in 3W ducks. Individual differences in the host's adaptive and innate immune responses to the viral infection would likely be such factors.

As we previously reported (30), a rapid production of neutralizing antibody in 3W ducks consistently led to a transient DHBV infection with smaller inocula than those used in this study, and differences in antibody production might be responsible for differences in virus spread in this study. The reduced ability to amplify cccDNA at early times after infection in 3W ducks would appear to contribute to the variability in outcome by limiting the early production of virus and possibly causing the spread of infection to be more sensitive to differences in the immune response.

ACKNOWLEDGMENTS

We thank Bai-Hua Zhang for excellent technical assistance.

This project was supported by grant CA84017 from the National Cancer Institute.

REFERENCES

1. **Beasley, R. P., C. Trepo, C. E. Stevens, and W. Szmuness.** 1977. The e antigen and vertical transmission of hepatitis B surface antigen. Am. J. Epidemiol. **105:**94–98.
2. **Ganem, D.** 1996. Hepadnaviridae and their replication, p. 2703–2737. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 2. Lippincott-Raven, Philadelphia, Pa.
3. **Hollinger, F. B.** 1996. Hepatitis B virus, p. 2739–2807. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 2. Lippincott-Raven, Philadelphia, Pa.
4. **Jilbert, A. R., T. T. Wu, J. M. England, P. M. Hall, N. Z. Carp, A. P. O'Connell, and W. S. Mason.** 1992. Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular involvement. J. Virol. **66:**1377–1388.
5. **Jilbert, A. R., D. S. Miller, C. A. Scougall, H. Turnbull, and C. J. Burrell.** 1996. Kinetics of duck hepatitis B virus infection following low dose virus inoculation: one virus DNA genome is infectious in neonatal ducks. Virology **226:**338–345.
6. **Jilbert, A. R., J. A. Botten, D. S. Miller, E. M. Bertram, P. M. Hall, J. Kotlarski, and C. J. Burrell.** 1998. Characterization of age- and dose-related outcomes of duck hepatitis B virus infection. Virology **244:**273–282.
7. **Lenhoff, R. J., and J. Summers.** 1994. Coordinate regulation of replication and virus assembly by the large envelope protein of an avian hepadnavirus. J. Virol. **68:**4565–4571.

8. **Lenhoff, R. J., and J. Summers.** 1994. Construction of avian hepadnavirus variants with enhanced replication and cytopathicity in primary hepatocytes. J. Virol. **68:**5706–5713.
9. **Lenhoff, R. J., C. A. Luscombe, and J. Summers.** 1998. Competition in vivo between a cytopathic variant and a wild-type duck hepatitis B virus. Virology **251:**85–95.
10. **Lenhoff, R. J., C. A. Luscombe, and J. Summers.** 1999. Acute liver injury following infection with a cytopathic strain of duck hepatitis B virus. Hepatology **29:**563–571.
11. **Mason, W. S., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. **36:**829–836.
12. **Mason, W. S., C. Aldrich, J. Summers, and J. M. Taylor.** 1982. Asymmetric replication of duck hepatitis B virus DNA in liver cells: free minus-strand DNA. Proc. Natl. Acad. Sci. USA **79:**3997–4001.
13. **Mason, W. S., M. S. Halpern, J. M. England, G. Seal, J. Egan, L. Coates, C. Aldrich, and J. Summers.** 1983. Experimental transmission of duck hepatitis B virus. Virology **131:**375–384.
14. **McMahon, B. J., W. L. Alward, D. B. Hall, W. L. Heyward, T. R. Bender, D. P. Francis, and J. E. Maynard.** 1985. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J. Infect. Dis. **151:**599–603.
15. **Miller, R. H., and W. S. Robinson.** 1984. Hepatitis B virus DNA forms in nuclear and cytoplasmic fractions of infected human liver. Virology **137:**390–399.
16. **Okada, K., I. Kamiyama, M. Inomata, M. Imai, and Y. Miyakawa.** 1976. e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N. Engl. J. Med. **294:**746–749.
17. **Pugh, J. C., and J. W. Summers.** 1989. Infection and uptake of duck hepatitis B virus by duck hepatocytes maintained in the presence of dimethyl sulfoxide. Virology **172:**564–572.
18. **Redeker, A. G.** 1975. Viral hepatitis: clinical aspects. Am. J. Med. Sci. **270:**9–16.
19. **Ruiz-Opazo, N., P. R. Chakraborty, and D. A. Shafritz.** 1982. Evidence for supercoiled hepatitis B virus DNA in chimpanzee liver and serum Dane particles: possible implications in persistent HBV infection. Cell **29:**129–136.
20. **Stevens, C. E., R. P. Beasley, J. Tsui, and W. C. Lee.** 1975. Vertical transmission of hepatitis B antigen in Taiwan. N. Engl. J. Med. **292:**771–774.
21. **Summers, J.** 1981. Three recently described animal virus models for human hepatitis B virus. Hepatology **1:**179–183.
22. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29:**403–415.
23. **Summers, J., P. M. Smith, and A. L. Horwich.** 1990. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. J. Virol. **64:**2819–2824.
24. **Summers, J., P. M. Smith, M. J. Huang, and M. S. Yu.** 1991. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. J. Virol. **65:**1310–1317.
25. **Tuttleman, J. S., C. Pourcel, and J. Summers.** 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell **47:**451–460.
26. **Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Buscher, R. Sprengel, R. Cattaneo, and H. Schaller.** 1987. Replication strategy of human hepatitis B virus. J. Virol. **61:**904–911.
27. **Wu, T. T., L. Coates, C. E. Aldrich, J. Summers, and W. S. Mason.** 1990. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. Virology **175:**255–261.
28. **Zhang, Y.-Y., and J. Summers.** 2000. Low dynamic state of viral competition in a chronic avian hepadnavirus infection. J. Virol. **74:**5257–5265.
29. **Zhang, Y.-Y., B. H. Zhang, D. Theele, S. Litwin, E. Toll, and J. Summers.** 2003. Single-cell analysis of covalently closed circular DNA copy numbers in a hepadnavirus-infected liver. Proc. Natl. Acad. Sci. USA **100:**12372–12377.
30. **Zhang, Y. Y., and J. Summers.** 2004. Rapid production of neutralizing antibody leads to transient hepadnavirus infection. J. Virol. **78:**1195–1201.